A STUDY ON THE PLATELET DYSFUNCTION IN CHRONIC LIVER DISEASE

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THE TAMIL NADU DR.MGR MEDICAL UNIVERSITY, CHENNAI, TAMIL NADU.

A STUDY

ON THE

PLATELET DYSFUNCTION

IN CHRONIC LIVER DISEASE

CERTIFICATE

This is to certify that this dissertation entitled "A STUDY ON THE PLATELET DYSFUNCTION IN CHRONIC LIVER DISEASE" submitted by Dr.G.Balasubramanian, to the faculty of Medical Gastroenterology, The Tamilnadu Dr.MGR Medical University Guindy, Chennai-600032, in partial fulfillment of the requirement for the award of DM., Degree Branch IV (Gastroenterology) is a bonafide work carried out by him under my direct supervision and guidance.

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INTRODUCTION

Liver dysfunction is frequently accompanied by a hemostatic defect.Bleeding is usually due to an anatomic lesion that is exacerbated by a hemostatic defect. Most patients with chronic liver disease bleed from complications of portal hypertension,esophageal varices, or gastritis and peptic ulcer disease.Portal hypertension also causes splenomegaly, with splenic sequestration of platelets and thrombocytopenia, which contributes to the hemostatic defect. Other causes of bleeding are variceal rupture,coagulation disorders due to multiple causes : decreased synthesis of clotting and inhibitor factors ; decreased clearance of activated factors , quantitative and qualitative platelet defects , hyperfibrinolysis and accelerated intravascular coagulation. Bleeding from the gastrointestinal tract is also an important determining factor of mortality in such patients.

In patients with chronic liver disease platelet dysfunction is an important contributing factor for bleeding which is not usually recognized in the routine clinical practice. Moreover tests assessing the functional status of platelets are seldom done in the laboratory due to various reasons.

This study is conducted from a tertiary referral centre for gastroenterological problems offering services free of cost to poor sections of the society.Patients who presented to our hospital with various complaints pertaining to chronic liver disease as well as those who were referred as chronic

liver disease were taken up for the study. Their blood samples were taken for analysis of platelet dysfunction and the tests were carried out in the Department of Biochemistry, Bharathi Women's College (Autonomous), Chennai.

AIM OF THE STUDY

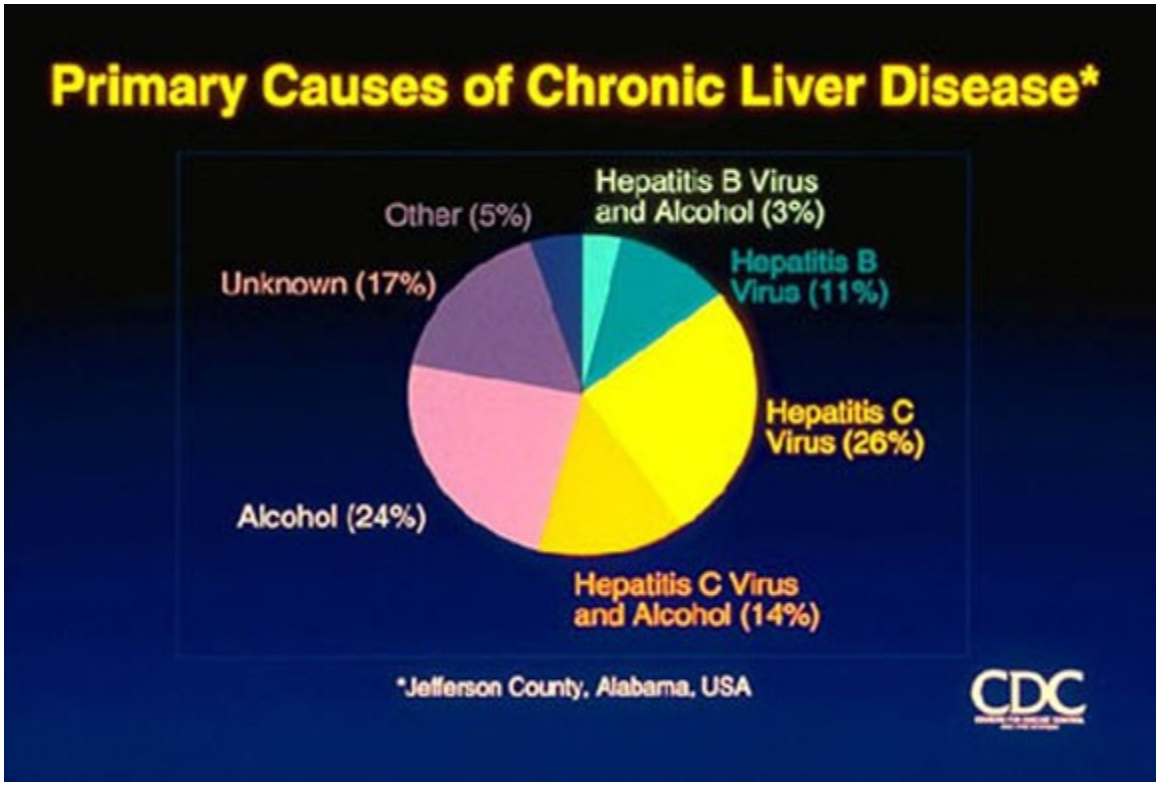
To assess the qualitative platelet function in patients with chronic liver disease.

1. To analyse the severity of platelet dysfunction in chronic liver disease patients with UGI bleed.
2. To compare the individual qualitative platelet function test in chronic liver disease patients.
3. To assess the contribution of platelet dysfunction in UGI bleed occurring in lesser grade of esophageal varices with relatively good liver function.
4. To determine the significance of regular assessment of qualitative platelet function in patients with chronic liver disease.

LITERATURE REVIEW

Introduction
Chronic Liver Disease

Chronic liver disease is an entity which can be due to different causes and resulting in altered hepatic function and eventually portal hypertension. Cirrhosis is the end stage of chronic liver disease and is characterized by fibrosis and conversion of normal liver architecture into structurally abnormal nodules.



In the western world, the most common cause of cirrhosis is infection with hepatitis C followed by alcohol abuse. But in our country, the most common cause of cirrhosis is alcohol abuse followed by hepatitis B. Other causes of cirrhosis are non-alcoholic steatohepatitis, autoimmune, metabolic, biliary and genetic disorders. The relative proportion of patients with "cryptogenic cirrhosis" is decreasing progressively.

Clinically cirrhosis can be divided into compensated and decompensated stages, each with different diagnostic, therapeutic and prognostic implications.

Compensated cirrhosis may be totally asymptomatic and diagnosed during routine biochemical testing or clinical or ultrasonographic abdominal examination. It may also become evident during abdominal surgery and or autopsy. Nonspecific asthenia, malaise, upper quadrant abdominal discomfort, or sleep disturbance may be the only complaint. On physical examination, spider angioma may be found, mostly on the trunk, face and upper limbs. Their number and size co-relate with disease severity. Palmar erythema involving the thenar and hypothenar eminences is the expression of a dense network of arterio-venous anastamosis.White nails may also appear. Common in male patient is hair loss on the chest and abdomen. Gynaecomastia and loss of libido may also occur. Petechaie and ecchymosis may be present as a result of thrombocytopenia and or prolonged prothrombin time. Duputyren's contracture involving the palmar fascia is particulary common in alcoholic patients. Hepatomegaly is very common, but liver size may also be normal or reduced. However, the consistency of the liver is invariably harder than normal. Splenomegaly is frequent and indicates presence of portal hypertension. Collateral circulation on the abdominal wall may develop as a consequence of portal hypertension.

In decompensated cirrhosis, signs of decompensation -ascites, variceal hemorrhage, jaundice and porto-systemic encephalopathy are found at

presentation in a proportion of patients, varying between 20 and 63%. Ascites by far most frequent sign of decompensation, being present in 80% of patients. On examination, patients with advanced cirrhosis may have malnutrition and muscle wasting, particularly in alcoholic cirrhosis. Jaundice and or ascites may appear. Other signs of decompensation are those of encephalopathy, as flapping tremor, bradylalia and mental state alterations. Frequently associated with encephalopathy and severe liver dysfunction is a sweetish smell of breath, fetor hepaticus. Hypotension and tachycardia due to hyperdynamic circulation secondary to portal hypertension may be present. Dyspnoea may also occur due to presence of large ascites, pleural effusion and or alteration of pulmonary circulation.

Diagnostic methods.

Liver biopsy still remains the gold standard for assessing the liver fibrosis and cirrhosis despite the limitation of sampling error and inter observer variability. However, eventhough definite tests such as histological diagnosis are important for patients and for the quantitative evaluation of treatment outcomes in clinical studies, non-invasive tests to estimate disease probability are necessary.

The presence of firm liver has been shown to be the most accurate sign of cirrhosis, with a diagnostic accuracy of 83% followed by the presence of collateral circulation(accuracy 77%).

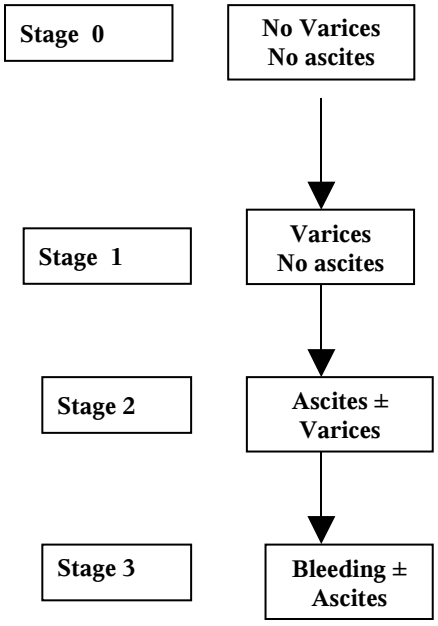
A value ≥1 for the ratio of aspartate aminotransferase to alanine aminotransferase (ALT/AST or AAR) has been proposed as a simple low cost predictor of cirrhosis in viral cirrhosis. A reduced platelet count (≤ 150 to ≤ 130 x 10^{9} / L) indicate the presence of cirrhosis. Prolonged prothrombin time also correlated with presence of cirrhosis. Recently, serum hyaluronate measurement has been shown to have an accuracy of about 90%, being particularly useful for excluding the presence of cirrhosis.

Ultrasonography is a useful tool for diagnosis as it may detect nodularity of liver surface, portal hypertension, splenomegaly and ascites.The sensitivity of ultrasonography in detecting portal hypertension has been shown to be 80% with a specificity of 100%, when assessing the lack of respiratory variation of splenic and superior mesenteric vein. The detection of liver nodular surface and detection of mean portal velocity shows 79% sensitivity and 80% specificity.

Upper digestive endoscopy has a definite role in the diagnostic workup as it may identify the presence of esophageal and /or gastric varices or congestive gastropathy. In compensated patients, the presence of esophageal varices has a sensitivity of 40% and a specificity of 99%.

In conclusion, a combination of clinical, biochemical, ultrasonographic and endoscopic data can establish the diagnosis of cirrhosis without liver biopsy with an accuracy of about 90 %.However when a precise determination of stage of fibrosis and degree of inflammation is required , liver biopsy is necessary.

CLINICAL STAGING



Treatment and Prevention

The treatment of compensated cirrhosis is essentially based on careful avoidance of alcohol and hepatotoxic drugs and early detection of initial signs of decompensation.An adequate nutrition regimen should include 1-1.5 gm protein/kg body weight .

Treatment of cirrhosis,particularly in the compensated phase of the disease , should be aimed at the interruption of fibrogenesis and the resorption of fibrosis.However, so far no drugs have been approved as antifibrotic agents in humans and the most effective way to eliminate or reduce hepatic fibrosis is to treat the underlying liver disease.A reduction in fibrosis has been reported in some patients with chronic infection with HCV treated with interferon alpha and ribavirin. Whether this effect may be reproduced also in cirrhotic patients is under investigation. No treatment has yet been shown to be effective in preventing the development or progression of esophageal varices.

The management of decompensated cirrhosis consists of treatment of complications and the only curative treatment is liver transplantation.

Prognosis of chronic liver disease

The outcome of cirrhosis is determined by 3 major factors

1. Survival time within the compensated phase
2. The intensity of transition from compensated to decompensated state
3. Survival while in the decompensated state.

The natural history of compensated cirrhosis is still poorly defined because patients are almost invariably free of symptoms and the diagnosis is usually prompted by casual discovery of abnormal liver function. Patients with compensated liver cirrhosis die after transition into decompensated cirrhosis. The 10 year survival rate for compensated patients is nearly 90%, while the median survival after decompensation is about 2 years. The progression to decompensation parallels the development and progression of portal hypertrension. The 10 year survival rate of decompensation is 10 %.

In patients with compensated cirrhosis, the development and enlargement of esophageal varices mark the progression of disease towards a more advanced state. When cirrhosis is diagnosed, the prevalence of varices ranges from 20% in compensated patients to 60 % in those presenting with ascites. The incidence of esophageal varices in newly

diagnosed cirrhosis is nearly 5 % per year.

Varices don't develop below a threshold HVPG of 10-12 mm of Hg. Above this threshold, the median time to development of varices and or bleeding or other complications of portal hypertension is about 4 years. Worsening liver function and continued exposure to alcohol are associated with an increase of HVPG with an increasing risk of developing varices.

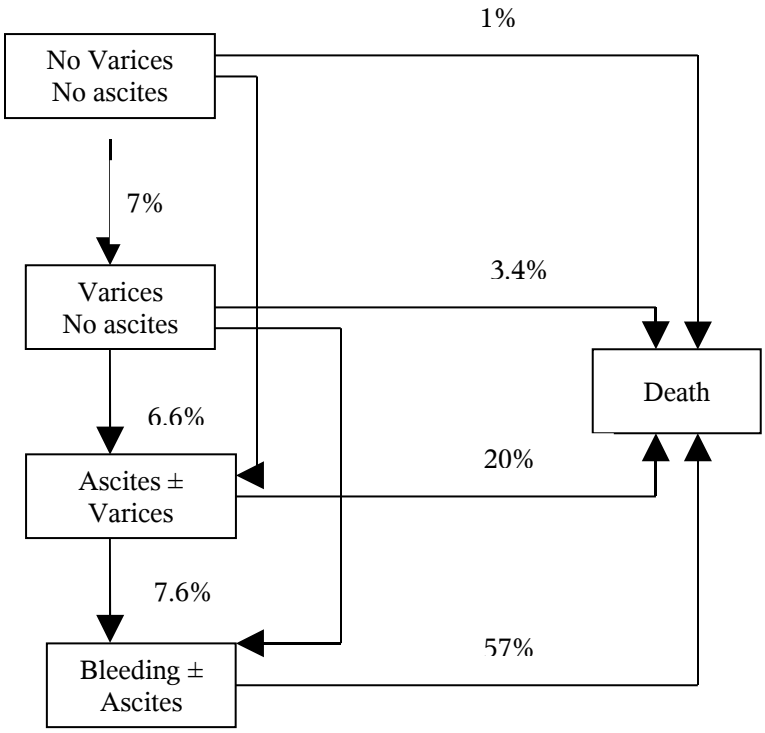
Once varices have developed, they increase in size from small to large before they eventually rupture and bleed. Median progression probability from small to large varices is 0.07 per year. Improvement in liver function and abstinence from alcohol may result in a decrease or even disappearance of varices, probably through a decrease in HVPG, as occurs for spontaneous or treatment induced HVPG reduction. Thus HVPG plays a key role in both the development and progression of varices.

Increasing size of esophageal varices associated with increasing bleeding risk (4 fold from absent to small varices, and 2 fold from small to large varices). A similar in risk has been shown for the development of ascites and mortality.

In clinical practice the size of the varices is the most widely used indicator of first variceal bleeding, because this risk is significantly reduced by prophylactic therapy in patients with medium to large varices. All patients with cirrhosis should be endoscopically screened for the presence of esophageal varices at the time of diagnosis of cirrhosis. Endoscopy should be repeated every 2-3 years in patients with cirrhosis without varices. In patients with compensated cirrhosis and small varices, endoscopy should be repeated every 1-2 years to detect the progression from small to large varices. In decompensated patients without varices or with small varices , endoscopy should be repeated yearly.

The median survival after first variceal haemorrhage is about one year (2 years if time zero is day 30 after the onset of bleeding) and after the development of ascites, it is 2 years.Encephalopathy and jaundice usually occur after bleeding or ascites and the median survival after the first appearance of encephalopathy or after the appearance of jaundice is therefore shorter than that for bleeding or ascites. The most frequent cause of death is bleeding, liver failure with hepatic coma, sepsis and hepatorenal syndrome.

Outcome of cirrhosis as related to clinical stage



Stages in the progression of cirrhosis

Prognostic Scores

Child's Classification

	A	B	C
S.Bilirubin	<2	2 to 3	>3
S.Albumin	>3.5	3 to 3.5	<3
Ascites	None	Easily controlled	Poorly controlled
Neurological disorder	None	Minimal	Advanced coma
Nutrition	Excellent	Good	Poor wasting

Numerous tests and formulas have been proposed , no method to assess functional hepatic reserve is uniformly accepted. Of these , the child pugh score is the best established .

The score is based on simple clinical variables (Encephalopathy and ascites) and blood test values (Serum bilirubin and albumin levels and prothrombin time)

CP grading of severity of liver disease was used to select a suitable candidate for shunt surgery who is likely to survive the operative

procedure .

Child pugh class a patients are operative candidates. If there is no ascites or encephalopathy , the bilirubin level is less than 3mg /dl , the albumin level greater than 3g / dl with an international normalized ratio less than or equal to 1.5 , the surgeon can operate on this patient with the expectation of a reasonable outcome . Child pugh class B patients may either class B improving towards to class A or class B moving towards class C.

In case of Child pugh class C patients, the only realistic operation is liver transplantation.

Numerous studies have failed to confirm its value in predicting morbidity and mortality after liver resection

Child Pugh Scoring System

	POINTS		
	1	**2**	**3**
Encephalopathy	None	1 to 2	3 to 4
Ascites	Absent	Slight/controlled by diuretics	At least moderate despite diuretics
Bilirubin	<2	2-3	>3
Albumin	>3.5	2.8-3.5	<2.8
Prothrombin (seconds prolonged)	<4	4-6	>6
INR	<1.7	1.7-2.3	>2.3
Cholestatic disease PBC/PSC	<4	4-10	>10

For primary biliary cirrhosis ,primary sclerosing cholangitis, or other cholestatic liver disease.

Grade A	5	-	6
Grade B	7	-	9
Grade C	10	-	15

Indices of liver dysfunction

Formulae for estimating the short –term prognosis of patients with alcoholic liver disease:

1. Composite clinical laboratory index (CCLI): Orrego and co-workers (1978) defined a group of parameters that correlate with mortality in hospitalized patients with alcoholic hepatitis. Calculation of the CCLI permits a linear estimate of acute mortality.
2. Maddrey's discriminant function (DF): Maddrey and co workers (1978) simplified the assessment of outcome of alcoholic liver disease by developing a discriminant function.

DF= 4.6 * (the difference between the patient's and control prothrombin time)+ serum bilirubin.

Patients with a DF greater than 32 have a 50% chance of dying during current hospitalization. This index offers the advantage of few variables and easy computation (and therefore easy to recall) but is relatively imprecise. Fifty percent of patients with a DF grater than 32 will survive the hospitalization.

The clinical tool is used most widely to determine prognosis in patients with alcoholic cirrhosis is the child turcotte pugh (CTP) classification. This simple classification system, which was designed specifically to assess the risk of requirement for portacaval shunt surgery in cirrhotic patients with variceal bleeding, has gained favor as a rapid method for determining the

prognosis of patients with various chronic liver diseases. The CTP classification is as effective as quantitative liver function tests and disease – specific prognostic models for determining short term prognosis in groups of patients awaiting liver transplantation . Despite its limitations , the CTP classification has been adopted widely for risk-stratifying patients with cirrhosis because of its simplicity and ease of use. Five year survival rates for patients with alcoholic cirrhosis decrease dramatically as the CTP class becomes higher at the time of clinical presentation.

The development of ascites, variceal bleeding, hepatic encephalopathy, spontaneous bacterial peritonitis or hepatorenal syndrome also has a significant impact on the prognosis of patients with alcoholic cirrhosis. The 5 year survival rate for persons in whom any of these complications develop is only 20% to 50% of that for patients with compensated cirrhosis. The most ominous complications are spontaneous bacterial peritonitis and rapid onset hepatorenal syndrome. Less than one half of the patients in whom spontaneous bacterial peritonitis develop can be expected to survive 1 year; the median survival of patients with hepatorenal syndrome is less than 2 weeks.

Other models that have been used to predict prognosis in patients with alcoholic cirrhosis are the proportional hazards model developed by poynard and colleagues, the beclere model and the prognostic model for end –satge liver disease (MELD) developed by investigators at the Mayo clinic. The beclere model which was developed from a database of 818 patients with alcoholic cirrhosis who were followed prospectively for 4 years, includes the serum bilirubin level , serum albumin level , patients age and presence or absence of hepatic encephalopathy . The MELD model, which originally was developed to assess short term prognosis in patients undergoing transjugular intrahepatic portosystemic shunt placement, includes the serum creatinine level , INR and serum bilirubin level. This model has been shown to be useful for predicting short term survival in groups of patients with various liver diseases.

Abstinence from continued excessive drinking is the most important predictor of survival in patients who survive an initial hospitalization for alcoholic cirrhosis. The rate of survival over the ensuing 2 years is 70% to 80% among patients who abstain or dramatically reduce their excessive drinking, compared with only 20% to 30% in those who continue to drink heavily.

Comparison studies concluded that three global indices of severity (CTP ,CCLI and maddrey index) all have approximately the same value for predicting mortality with the exception maddrey index ,which is slightly less reliable.

Child's grade is used to assess hepato cellular function in cirrhosis . Every patient should be assigned a grade.

It is the most important predictor of the likelihood of bleeding (in chronic liver patients who are having oesophageal varices). It correlates with variceal size and with the presence of endoscopic red signs and with the response to treatment.

Child's grading and hospital **death at index bleed**

Grade A	5%
Grade B	18%
Grade C	68%
Total	50%

PLATELETS

Platelets (thrombocytes) are anucleate cells seen in the blood which are derived from the megakaryocytes in the bone marrow. It is of 2-4 μm in size and is of irregular or oral disc in shape. Normal platelet count in an adult is 2-4 lakh/cu mm.

Platelets are surrounded by a plasma membrane with a thick

glycoprotein coat,which is responsible for their adhesive properties. The cytoplasm of platelets contain microtubules, mitochondria, glycogen, endoplasmic reticulum and 3 major types of membrane bound vesicle, ie, alpha, delta and lambda granules. Microtubules lies beneath the plasma membrane and associated with actin filaments, myosin and other proteins related to cell contraction. Alpha granules are the largest granules (500 nm) and contains platelet derived growth factor (PDGF), fibrinogen and other substances.

Platelets remain in the blood circulation for about 10 days. The megakaryocytes, giant cells in the bone marrow, form platelets by pinching off bits of cytoplasm and extruding them into the circulation. Between 60 and 75 % of the platelets that have been extruded from the bone marrow are in the circulating blood, and the remainder are mostly in the spleen. They are removed mainly by the splenic macrophages. Splenectomy increases platelet count.

The plasma membrane of platelets contain receptors for collagen, ADP, vessel wall von Willebrand factor and fibrinogen. Platelets normally circulate in an unstimulated disk shaped form. During hemostasis or thrombosis, they become activated and help to form hemostatic plugs or thrombi. 3 major steps are involved (1) adhesion to exposed collagen in blood

vessels,(2) release of the contents of their granules and (3) aggregation.

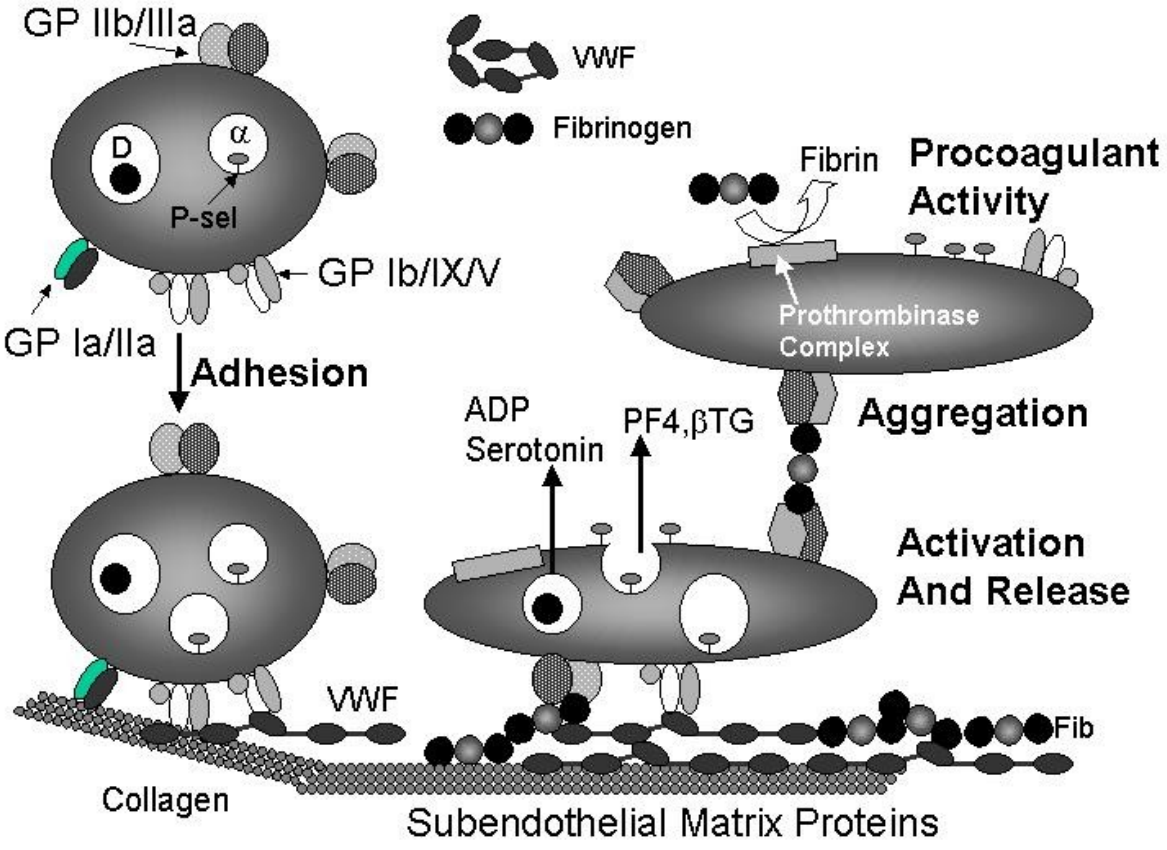
Platelets adhere to collagen via specific receptors on the platelet surface, including the glycoprotein complex GPIa-IIa (α2β1 integrin), in a reaction that involves von Willebrand factor. This is a glycoprotein, secreted by endothelial cells into the plasma, which stabilizes factor VIII and binds to collagen and subendothelium. Platelets bind to von Willebrand factor via a glycoprotein complex (GPIb-V-IX) on the platelet surface; this interaction is especially important in platelet adherence to the subendothelium under conditions of high shear stress that occur in small vessels and stenosed arteries.

Platelets adherent to collagen change shape and spread out on the subendothelium. They release the contents of their storage granules; secretion is also stimulated by thrombin.

Thrombin, formed from the coagulation cascade, is the most potent activator of platelets and initiates platelet activation by interacting with its receptor on the plasma membrane. The further events leading to platelet activation are examples of transmembrane signaling.The interaction of thrombin with its receptor stimulates the activity of an intracellular phospholipase Cβ. This enzyme hydrolyses the membrane phospholipids phosphotidyl 4, 5 biphosphate (PIP_2) to form 1, 2 diacylglycerol and 1,4,5

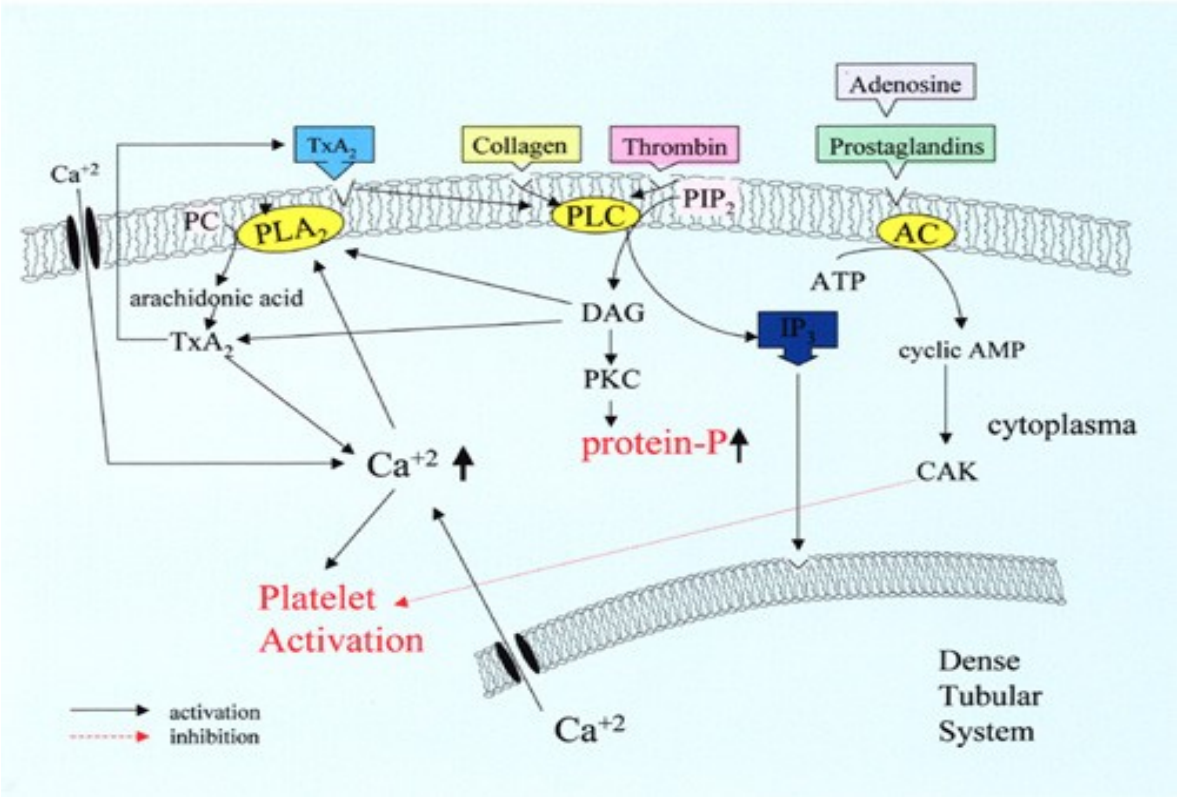
inositol triphosphate.

Diacylglycerol stimulates protein kinase C, which phosphorylates the protein pleckstrin. This results in aggregation and release of the contents of the storage granules. ADP released from dense granules can activate platelets, resulting in aggregation of additional platelets. IP3 causes release of Ca^2 into the cytosol mainly from the dense granular system (or residual smooth endoplasmic reticulum from the megakaryocyte), which then interacts with calmodulin and myosin light chain kinase, leading to phosphorylation of the light chains of myosin. These chains then interact with actin, causing changes of platelet shape.



Collagen-induced activation of a platelet phospholipase A_2 by increased levels of cytosolic Ca^{2+} results in liberation of arachidonic acid from platelet phospholipids, leading to the formation of thromboxane A_2, which in turn, in a receptor-mediated fashion, can further activate phospholipases C, promoting platelet aggregation.

Activated platelets, besides forming a platelet aggregate, are required, via newly expressed anionic phospholipids on the membrane surface, for acceleration of the activation of factors X and II in the coagulation cascade.



All of the aggregating agents, including thrombin, collagen, ADP, and others such as platelet-activating factor, modify the platelet surface so that fibrinogen can bind to a glycoprotein complex, GPIIb-IIIa, on the activated platelet surface. Molecules of divalent fibrinogen then link adjacent activated platelets to each other, forming a platelet aggregate.

Clinical manifestations of disorders of primary Hemostasis.

(platelet defect)

Manifestations	Defects of primary Hemostasis (Platelet Defect)
Onset of bleeding after trauma	Immediate
Sites of bleeding	Superficial – skin , mucous membranes,nose ,gastrointestinal and genitourinary tracts.
Physical findings	Petechiae , ecchymoses
Family history	Autosomal dominant
Response for therapy	Immediate: local measures effective

Evaluation of Platelet Function

Platelet function in chronic liver disease

Platelet abnormalities found in patients with liver disease include reduced platelet count and functional disorders such as impaired aggregation and adhesion.

Thrombocytopenia in liver disease often is caused by sequestration in enlarged spleen.Shortened survival of platelets and fibrinogen in liver disease has been found. Although fibrinogen survival improved after the administration of heparin ,such an effect was not found on platelet , indicating that this would be dependent on mechanisms other than thrombin mediated platelet consumption.

In addition to the sequestration of platelets in the spleen, an impaired

synthesis of thrombopoietin may contribute to the thrombocytopenia seen in patients with liver disease.

In patients with alcohol related liver disease, there may be a direct toxic effect of ethanol on megakaryocyte proliferation, contributing to thrombocytopenia.

Platelet dysfunction including impaired platelet aggregation of adenosine diphosphate, epinephrine, thrombin, ristocetin in citrated platelet rich plasma has been described in patients with chronic liver disease. In patients with a bleeding time more prolonged than expected from the platelet count, an abnormal platelet aggregation appeared to correlate with prolongation of bleeding time. The functional abnormalities have been attributed to an inhibitory effect of elevated plasma FDP s on platelet aggregation or the presence of abnormal high density lipoproteins. Moreover data have been reported indicating that platelet functional defect is intrinsic and not inducible by short term incubation of normal platelets in plasma from cirrhotic patients. Disturbances of prostaglandin metabolism, increased cholesterol content of the platelet plasma membrane or an impairment of platelet transmembrane signaling mechanism may also contribute to the defect.

Role of nitric oxide in the pathogenesis of primary hemostatic disorders associated with chronic liver disease.

Nitric oxide is a powerful endothelium derived vasodilator that, in addition to the relaxation effect on vascular smooth muscle, is a potent inhibitor of platelet function. It has been demonstrated that nitric oxide synthesized by the constitutive nitric oxide synthase in both platelets and vascular endothelium, inhibits platelet aggregation and adhesion, exerting its action through activation of the soluble guanylyl cyclase and elevation of cyclic GMP. Under pathological conditions, platelet function may also be

modified as a result of the expression of the inducible nitric oxide synthase. Recent studies suggest that increased nitric oxide formation plays a role in the pathogenesis of the hemodynamic abnormalities associated with portal hypertension.

METHODS

MATERIALS AND METHODS

The study was conducted between January 2005 and March 2007 in the Government Peripheral Hospital, Annanagar. It prospectively enrolled consecutive 60 patients with chronic liver disease. The inclusion and exclusion criteria were as follows.

Inclusion Criteria

1. Patients detected to have chronic liver disesase by investigations like ultrasonography, biochemical tests and endoscopy.
2. Patients presenting with history suggestive of chronic liver disease and confirmed by various investigations

.

Exclusion Criteria

1. Patients with recent surgery, cardiopulmonary bypass, advanced malignancy, sepsis, disseminated intravascular coagulation, renal dysfunction.
2. Patients on anticoagulants, antiplatelet drugs.

3. Patients with disorders of blood like polycythemia vera, essential thrombocythemia, thrombocytopenia, von Willebrand's disease

Data was collected at the time of presentation to our hospital.

Detailed examination of the patients was carried out including general physical examination for any evidence of icterus, anemia and stigmata of chronic liver disease. Cardiovascular, respiratory and neurological examination was carried out to look for any evidence of systemic involvement. A detailed abdominal examination was done to look for ascites, hepatomegaly, splenomegaly and also abdominal wall collaterals. All the patients were investigated with hemogram, ESR, total and differential leucocyte counts, platelet count, blood sugar, blood urea, serum creatinine, liver function tests which include S.bilirubin (total & direct),SGPT, SGOT, total proteins, albumin. All the patients underwent ultrasonography and UGI endoscopy After confirmation of chronic liver disease blood samples were taken for qualitative platelet analysis which was carried in the Department of Biochemistry, Bharathi Women's College (Autonomous), Chennai.

Platelet function studies that were assessed were tests for platelet adhesion (ADP-induced adhesion & collagen-induced adhesion), tests for

platelet aggregation (ADP-induced aggregation and collagen-induced aggregation) and tests for estimation of nitric oxide (NO) production. (In mM of total nitrite /mg of PRP protein).

Isolation of platelets

The method of Aster and Jandl, 1964 was adopted for the isolation of platelets. Briefly, 10 ml of blood was collected with acid citrate dextrose (2.5 g of trisodium citrate, 2 g of dextrose and 1.5 g of citric acid in 100 ml of distilled water) anticoagulant in the ratio 9:1. The blood was then centrifuged at 160 x g for 10 min to obtain platelet rich plasma (PRP).The PRP thus obtained was subjected to centrifugation at 300 x g for 5 minutes to pellet out the platelets. The remaining plasma devoid of platelets is the platelet poor plasma (PPP) and this is used as the control. The pelleted platelets were then washed repeatedly with washing buffer containing 0.113M NaCl,4.3 mMK_2HPO4, 4.3 mM Na_2HPO4, 24.4 mM NaH_2PO4 and 5.5 mM glucose , pH 6.5 to obtain erythrocyte free platelet suspension and the purity was confirmed by microscopic examination. The washed platelet pellet was then suspended in platelet storage buffer containing 0.109 M NaCl, mM K_2HPO4, 4.3 mM Na_2HPO4, 16 mM NaH_2PO4, 8.3mM NaH_2PO4 and 5.5 mM glucose, pH 7.5 and stored at 4^o C until further analysis.

Assay of platelet adhesion

Platelet adhesion was assayed by the method of Bellavite et al., 1994. The harvested platelets were gently suspended in buffer containing 145 mM NaCl, 5 mM KCl, 10 mM HEPES ,0.5 mM Na_2HPO4, 6 mM glucose , 0.2% human serum albumin in 1 litre distilled water with pH 7.4.Microplate coating was performed overnight at 4^0 C by adding 100µl /well of human plasma diluted 1:1 at PBS or 20µl/ml collagen in 0.9% NaCl. Immediately before use, the plates were washed 2 times with 0.9% NaCl to avoid non-specific binding of platelets. Immediately after coating and washing, the wells were supplemented with 25µl/of agonist 4µM ADP/2µM collagen / fresh plant extract. The plate was then brought to 37^oC and 50µl/of prewarmed platelet ($2.5x10^5$ platelets) at 37^o C was added to each well. The plate was incubated for 10 min at 37 C. At the end of incubation the wells were washed twice with 0.9% saline (to remove non-adhered platelets) and were rapidly supplemented with 150µl of 0.1% Triton X-100.After incubation at room temperature for 1 hour the reaction was stopped and the colour was developed by the addition of 100µl of 2 N NaOH. The p-nitro phenol produced by the reaction was measured with Qualigens-SR 601 ELISA strip reader at 405 nm against a platelet free blank. The enzyme activity is the measure of platelet adhesion. The percentage of adherent cells was calculated on the basis of a

standard curve obtained with known number of platelets. The results were expressed as % adhesion.

Platelet aggregation assay

Spectrophotometric method

Platelet aggregation was assayed by the method sited by **Bellavite et al.,(1994)**.The method is based on the measurement of turbidity at 620 nm.Briefly ,1.0 ml of the test agonist 4µM ADP/2µM collagen in buffer A (145 mM NaCl, 5 mM KCl,10 mM HEPES ,0.5 mM Na_2HPO4, 6 mM glucose , 0.2% human serum albumin in 1 litre distilled water, pH 7.4.) supplemented with 3 mM $CaCl_2$ and 3 mM $MgSO_4$ was added with 1.0 ml of the platelet suspension incubated priorily at 37^o C and the increase in transmission at 620 nm was followed over time with Systronics Visible Spectrophotometer-106.The percentage of aggregation was calculated on the basis of a standard curve obtained with known number of platelets.The results were expressed as % aggregation.

ELISA method

Platelet aggregation was assayed by the method sited by Bellavite et al., (1994).The method is based on the measurement of turbidity at 620 nm directly into microplates. Briefly ,the wells were supplemented with 100µl of the test agonist 4 µM ADP/2µM collagen in buffer A (145 mM NaCl, 5 mM

KCl,10 mM HEPES,0.5 mM Na_2HPO4, 6 mM glucose , 0.2% human serum albumin in 1 litre distilled water, pH 7.4.) supplemented with 3 mM $CaCl_2$ and 3 mM $MgSO_4$ .After the addition of 200 ul of the platelet suspension ,the strip was incubated at 37° C and the decrease in absorbance at 620 nm was followed over time with Qualigens-SR 601 ELISAstrip reader.The percentage of aggregation was calculated on the basis of a standard curve obtained with known number of platelets.The results were expressed as % aggregation.

Platelet secretion assay

0.5 ml of the agonist solution (4μM ADP) was added with 0.5 ml of test platelet suspension and for control; samples were added with distilled water devoid of agonist and incubated at 37° C for 5 minutes. The reaction was terminated by mixing the sample with 0.5 ml of ice cold 0.633 M formaldehyde in 0.05 M EDTA. The formaldehyde fixed aliquots were kept on ice for upto 60 minutes,. The aliquots were the n centrifuged at 12000 x g for 2 minutes at room temperature. The supernatants obtained contain, the substances excreted from the platelets(S_{test}) and the substances in the water excreted platelets($S_{control}$).Additional aliquots for the determination of total contents ($T_{control}$) of secretable substances. i.e., non-centrifuged samples were

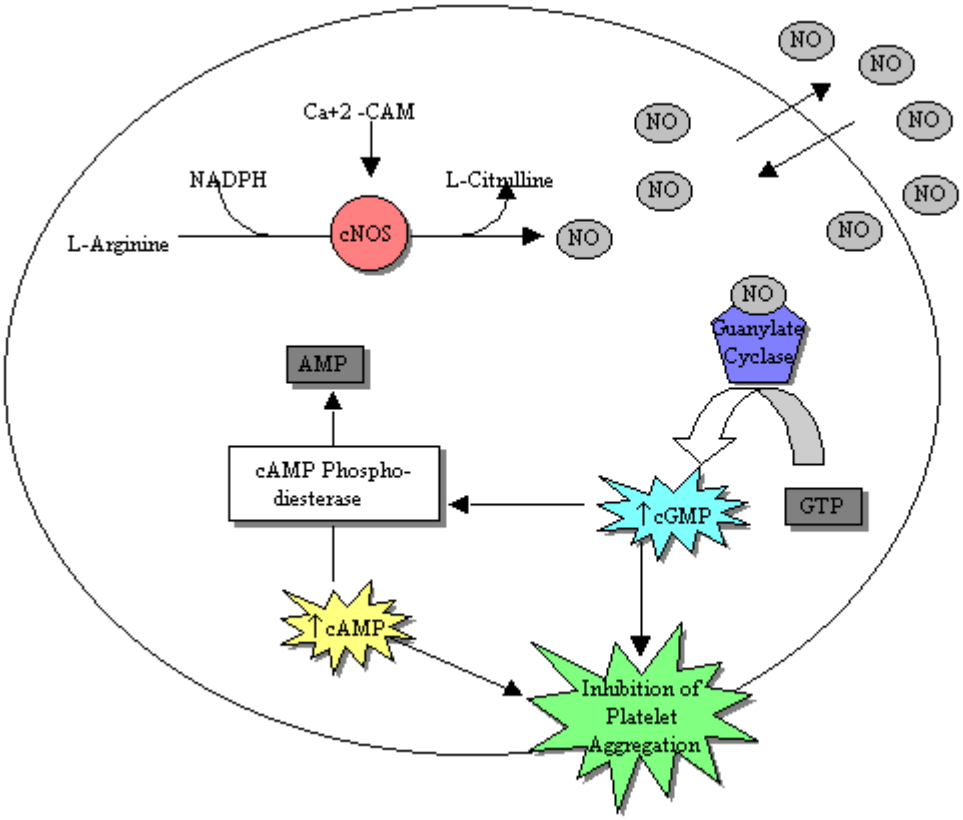
also prepared simultaneously. The supernatants were treated with 0.3 ml of 12N HCl and the fluorescent intensities of the samples were determined at an excitation wavelength of 303 nm and emission wavelength of 345 nm with ELICO-SL 174 Spectroflourimeter. Percentage secretion was calculated by the formula:

Percentage secretion=$[(S_{test}-S_{control})/(T_{test}-T_{control}]x\ 100$

Where, S- amount of the substance in the supernatants

T-Total content

Estimation of nitric oxide



Estimation of nitric oxide in terms of total nitrite was based on the method of Griess (1879) modified by Fiddler (1977). This method is based on the Griess reaction in neutral solution which on exposure to the intermediates in the NO/ O_2 reaction form an azo dye with NEDD and sulphanilamide. Nitrite forms significant amount of a nitrosating species only under acidic condition .The nitrate present in the sample cannot react with Griess reagent and so prior to

the reaction nitrate is reduced to nitrite and then made to react with Griess reaction. The colour intensity of the azo dye measured at 520 nm is directly proportional to nitric oxide content in the biological sample

Reagents

1. Solution A :0.1% Naphthyl ethylene diamine dihydrochloride-100 mg of Naphthyl ethylene diamine dihydrochloride was dissolved in distilled water.
2. Solution B: 1% Sulphanilamide-1 g of Sulphanilamide was dissolved in 100 ml 5 % phosphoric acid.
3. Griess reagent - Equal volumes solution A and solution B serves as griess reagent.
4. 5 % phosphoric acid—6.5 ml of 85% phosphoric acid was diluted to 100 ml with distilled water,
5. 35% Sulphosalicylic acid - 35 g of sulphosalicyclic acid was dissolved in 100 ml distilled water.
6. Reducing agent-Tin balls soaked in 6 N HCl.
7. Standard Sodium nitrite-0.005 mM/ml

Procedure

0.7 ml of PRP was treated with 0.3 ml of sulphosalicylic acid and mixed well. Samples were vortexed for every 5 minutes for the total period of 30 minutes at room temperature and centrifuged at 3000 rpm for 30 minutes. 0.5 ml of the supernatant was mixed with 1 ml of 6 N HCl containing 3-4 tin balls. The contents were incubated at room temperature for 30 minutes with intermittent shaking in a dark environment. Then 1 ml of Griess reagent was added and allowed to stand for 20 minutes at room temperature protected from light. The color intensity developed was read at 520 nm using Systronics 106-mono beam spectrophotometer. Aliquots of standard were also treated similarly.

The values were expressed as mM of total nitrite/mg of PRP protein.

DISCUSSION

Platelet dysfunction is an important cause of bleeding in patients with chronic liver disease. Similar observations were obtained in several other studies.

Our study group consists of 60 patients .Ther is a male predominance in the study (31 males versus 29 females)

The whole study group(N=60) is characterized into two groups.

Group1 - Those who presented with upper gastrointestinal bleed(N=31)

Group 2-Those who presented without bleed (N=29)

The two groups contain almost equal number of patients.Our hospital being a tertiary referral centre; nine patients were referred as chronic liver disease. In 60 patients majority of the patients (ie, 50) had ascites of varying grades.24 patients were jaundiced.7 patients had sleep disturbances.None of the patients had frank encephalopathy.

The most common etiology of chronic liver disease patients was alcohol abuse.

All the patients' undergone ultrasonography and all of them showed various features of chronic liver disease.

Those patients with other medical illnesses like malignancy, thrombocytopenia etc were excluded.

The patients had undergone routine biochemical tests and in addition

qualitative platelet function studies. The function studies done were as Percentage of platelet aggregation, platelet adhesion and estimation of platelet nitric oxide. On comparing the platelet function studies it was found that all the three funtions are defective in chronic liver disease patients. Among the functions, estimation of nitric oxide appeared to be the most sensitive marker. There is a 35 % reduction in the % of platelet aggregation and 40% reduction in the % of platelet adhesion in patients with chronic liver disease compared to that of normal individuals. A study conducted in 24 patients with portal cirrhosis and compared with platelet aggregation in 14 normal subjects by Dr.Ballard H.S showed similar finding.

All the three platelet function tests were abnormal in both the bleeding and non-bleeding group of patients with chronic liver disease .This finding was observed in studies done by Dr.Calabrese S and Dr.Giansante C.

Out of the 31 patients who presented with upper gastrointestinal bleeding,26 patients(83%) showed esophageal varices of varying grades ranging from 2 to 4 on endoscopy.In the remaining 5 patients(16%),2 patients showed grade 1 varices and three patients showed no varices .

Platelet function tests of the three patients who had no varices were compared with those who have Grade 2-4 varices. In patients who presented with upper gastrointestinal bleed and absent varisces on endoscopy,the

platelet function tests show s abnormalities which is similar to that seen in patients with GI bleed and varices. This implies that platelet dysfunction place a definite role in UGI bleed in chronic liver disease patients who have no varices.

The severity of chronic liver disease using Child's-Pugh scoring system was defined.15 patients belonged to Child's A, 16 patients in Child's B and 29 patients in Child's C. The platelet function tests were compared among the patients belonging to the above three groups. No correlation was found between the severity of the chronic liver disease and platelet dysfunction.

CONCLUSION

Qualitative function of platelets is defective in all patients with chronic liver disease

1. There is no correlation between platelet dysfunction and bleeding in patients with chronic liver disease.
2. Estimation of nitric oxide in plasma appears to be a more sensitive marker of platelet dysfunction among the various tests for platelet dysfunction.
3. Platelet dysfunction is a contributing factor in UGI bleed occurring in lesser grade of esophageal varices with relatively good liver function.
4. At present there is no definite role for assessment of qualitative platelet function in the routine management of patients with chronic liver disease.

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Proforma

1. Name:

2. DDHD Number:

3. Age:

4. Sex:

5. Symptoms:

Present History

(0=absent, 1=present)

6. Asymptomatic

7. Generalised weakness:

8. Right upper quadrant abdominal discomfort:

9. Yellow urine/yellow sclera/yellow discoloration of body:

10. Haemetemesis / melaena:

11. Abdominal distension:

12. Swelling of legs:

13. Abdominal swelling:

General Examination (0=absent, 1=present)

14. Icterus:

15. Pallor:

16. Cyanosis:

17. Lymphadenopathy:

18. Evidence of malnutrition:

19. Palmar erythema

20. Duputryren’s contracture:

21. Spider naevi:

22. White nails:

23. Ankle edema

24. Flapping tremor

25. Cyanosis:

26. Hair loss:

27. Gynaecomastia:

Abdominal Examination (0=absent, 1=present, 2=cannot be tested))

28. Hepatomegaly:

29. Splenomegaly:

30. Ascites:

31. Abdominal wall collaterals:

CNS Examination

32. Mental State Examination

33. Bradylalia

CVS Examination

Respiratory System Examination

Investigations (0=normal,1=abnormal,2=not done)

34. Hemoglobin:

35. Platelet count:

36. Liver function tests:

1. S.Bilirubin(Total & Direct):

2. SGOT:

3. SGPT:

4. Total protein:

5. S.Albumin

36. Ultrasonogram

1. Liver size , echotexture and nodularity
2. Portal vein diameter and portal flow velocity
3. Respiratory variation of splenic and superior mesenteric vein

37. Endoscopy

1. Esophageal varices
2. Gastric varices
3. Congestive gastropathy